

FORM PTO-1390 (Modified)
(REV 11-2000)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

DRE-0057

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

09/856937

INTERNATIONAL APPLICATION NO.
PCT/US99/28403INTERNATIONAL FILING DATE
30 November 1999PRIORITY DATE CLAIMED
30 November 1998

TITLE OF INVENTION

Methods and Kits for Identifying Individuals at Risk of Developing Osteoporosis

APPLICANT(S) FOR DO/EO/US

SPOTILA, Loretta D.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ has been communicated by the International Bureau.
 - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☐ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)). - **unexecuted**
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with 37 CFR 1.821.
20. ☐ A second copy of the published international application under 35 U.S.C. 122(b).
21. ☐ A second copy of the English language translation of the international application.
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

- 1) Courtesy copy of International Application;
- 2) Copies of Written Opinion and Response thereto;
- 3) Return post card

"Express Mail" Label No. **EL780366126US**
Date of Deposit **May 30, 2001**

I hereby certify that this paper is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Box PCT, Washington, D.C. 20231.

By Deborah Ehret
Typed Name: Deborah Ehret

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 09/856937)	INTERNATIONAL APPLICATION NO. PCT/US99/28403	ATTORNEY'S DOCKET NUMBER DRE-0057
--	--	---

24. The following fees are submitted:				CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :					
<input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1000.00					
<input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00					
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00					
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00					
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00					
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$860.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).				\$0.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	4 - 20 =	0	x \$18.00	\$0.00	
Independent claims	2 - 3 =	0	x \$80.00	\$0.00	
Multiple Dependent Claims (check if applicable). <input type="checkbox"/>				\$0.00	
TOTAL OF ABOVE CALCULATIONS =				\$860.00	
<input checked="" type="checkbox"/> Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				\$430.00	
SUBTOTAL =				\$430.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).				\$0.00	
TOTAL NATIONAL FEE =				\$430.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). <input type="checkbox"/>				\$0.00	
TOTAL FEES ENCLOSED =				\$430.00	
Applicant, Drexel University, is entitled to small entity status. It is a non-profit organization.				Amount to be refunded	\$
				charged	\$

- a. ☒ A check in the amount of **\$430.00** to cover the above fees is enclosed.
- b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **50-1619** A duplicate copy of this sheet is enclosed.
- d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Jane Massey Licata, Reg. No. 32,257
Kathleen A. Tyrrell, Reg. No. 38,350
Licata & Tyrrell P.C.
66 E. Main Street
Marlton, New Jersey 08053

Telephone: (856) 810-1515
Facsimile : (856) 810-1454

Kathleen A. Tyrrell
SIGNATURE
Kathleen A. Tyrrell
NAME
38,350
REGISTRATION NUMBER
May 30, 2001
DATE

**METHODS AND KITS FOR IDENTIFYING INDIVIDUALS AT RISK
OF DEVELOPING OSTEOPOROSIS**

INTRODUCTION

5 This invention was made in the course of research sponsored by the National Institutes of Health. The U.S. Government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

10 Osteoporosis is a disabling condition characterized by low bone density and fragile bones. The term "osteoporosis", encompasses diseases of many different etiologies that all result in a reduction in the mass of bone per unit volume. Osteoporosis can be seen without association with other disease (idiopathic) and also in
15 conjunction with other diseases (e.g., rheumatoid arthritis, diabetes, calcium deficiency). The decreased bone mass characteristic of the disease is not due to a decrease in the ratio of mineral to organic phase of bone, nor to any known abnormality in the mineral or organic
20 matter of bone. Histological changes seen include a decrease in cortical thickness and a decrease in the number and size of the trabeculae of cancellous bone. Osteoporosis is the most common of all metabolic bone diseases and is seen commonly in the elderly.

25 Since bone mass is decreased in osteoporosis, the rate of bone resorption exceeds that of bone formation. There is a normal age-related loss of bone mass in adults. However, this age-related loss of bone begins earlier and proceeds more rapidly in women. The reasons for the age-
30 related bone loss are not known, although risk factors identified have included race (white women being at greatest risk), sex (females), sedentary lifestyle, and less muscular development. Other factors associated with

-2-

risk of developing osteoporosis include early menopause, smoking, and excessive alcohol consumption.

Diagnosis of osteoporosis is most often done in conjunction with a study of bone density by radiography.

5 Although clinical laboratory tests such as levels of calcium and phosphorus in blood can be examined, these measures are usually normal in osteoporotic patients. Only about 20% of postmenopausal women with osteoporosis exhibit hypercalciuria, or increased excretion of calcium in urine.
10 Therefore, such laboratory findings are not indicative of the presence of disease, and clearly would not be indicative of risk of developing disease. To date, the prediction of risk of developing disease relies on family history of the disease. However, no genetic test is
15 currently available to screen individuals.

The ethnic differences shown to exist in the propensity to develop osteoporosis have led researchers to believe that genetic factors play a dominant role in the etiology of this disease. Several genes have been shown to
20 be associated with low bone density and research has focused on identifying those genes that may act as markers of disease. Common allelic variations of the vitamin D receptor gene have been found to be associated with decreased bone density in certain populations, including
25 premenopausal women and young girls (Wood, R.J. and Fleet, J.C. *Ann. Rev. Nutrit.* 1998 18:233-258). Bone mineral density has also been associated with genetic variation in the estrogen receptor gene, both by itself and in conjunction with variations in the vitamin D receptor gene
30 (Willing et al. *J. Bone Min. Res.* 1998 13:695-705). In Japanese women, the HLA-A*24-B*07-DRB*01 halotype has been linked to low peak bone mass (Tsuiji et al. *Hum. Immunol.* 1998 59:243-249). A variant of the gene encoding transforming growth factor-beta 1 has also been associated
35 with low bone mass in osteoporotic women and with low bone

mass and increased bone turnover in normal women (Langdahl et al. *Bone* 1997 20:289-294). A polymorphism of the COL1A1 gene has been identified as a potential marker for low bone mass and vertebral fracture in women (Grant et al. *Nat.*

5 *Genet.* 1996 14:203-205). However, none of these studies has examined the relationship between tumor necrosis factor alpha receptor 2 gene and osteoporosis or bone mass in women.

Devoto et al. (*Eur. J. Hum. Genet.* 1998 6:151-157)
10 determined that there was a gene or genes on chromosome 1 of humans that was linked to low bone density. However, there are many potential genes on this chromosome that could be involved in the etiology of low bone density.

It has now been found that individuals at risk of
15 developing osteoporosis can be identified by assessing the genotype of the tumor necrosis factor alpha receptor gene in a sample of DNA obtained from the individual. A polymorphism in the gene on chromosome 1 for tumor necrosis factor alpha receptor 2 has now been shown to be associated
20 with low bone density.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a method of screening individuals for risk of developing osteoporosis. This method is based on assessment of the
25 genotype of the tumor necrosis factor alpha gene in a DNA sample obtained from the individual. The presence of polymorphisms at nucleotides 593, 598 and 620 in exon 10 of the tumor necrosis factor alpha 2 receptor gene is associated with low bone density.

30 Another object of the present invention is to provide a kit for screening individuals for risk of developing osteoporosis which comprises a means for assessing the genotype of the tumor necrosis factor alpha 2 receptor gene in an individual.

DETAILED DESCRIPTION OF THE INVENTION

In the present invention a method is provided for screening individuals for the presence of a polymorphism in the tumor necrosis factor alpha receptor 2 (TNFR2) gene.

5 The presence of this genotype in individuals has now been shown to be associated with low bone density and the risk of developing osteoporosis. The association between TNFR2 gene and low bone density was based on the observation that there is a polymorphism in this gene at a particular region
10 of the gene sequence. This polymorphism was identified by Kaufman et al. (*Hum. Genet.* 1994 94:418-422), however, no characterization of its nature or examination of the relationship with bone density was disclosed. In the present invention, a relationship of this polymorphism with
15 bone density has now been identified.

In these experiments, two polymorphic sites within the TNFR-2 gene, including a microsatellite repeat within intron 4 and a three nucleotide variation in the 3'untranslated region of the gene were assayed in three
20 groups of individuals. Test group 1 was comprised of individuals from families used for linkage analysis of the low BMD (mean bone density) trait. Test group 2 was made up of unrelated individuals from an osteoporosis clinic. The third group was made up of unrelated individuals from a
25 general clinic population with no testing for or knowledge of their bone status. This third group was used as reference for allele frequency comparisons. The composition and mean bone density Z-scores for test groups 1 and 2 are shown in Table 1.

Table 1: Results of ANOVA among the males and females comprising the two test groups for age, body mass index and bone density

	GROUP 1		GROUP 2		P Value	F ratio (df)
	Male	Female	Male	Female		
Number	22	44	12	81		
Age (yrs \pm SE)	56.2 (2.5)	57.3 (1.9)	43.7 (3.8)	52.6 (1.5)	0.011	3.846 (3,153)
Body Mass Index (\pm SE)	28.4 (1.3)	25.9 (0.9)	25.0 (1.7)	24.8 (0.6)	0.084	2.259 (3,149)
Mean BMD Z-score (\pm SE)						
L2-L4	-0.56 (0.38)	-1.17 (0.21)	-1.02 (0.49)	-0.99 (0.14)	0.442	0.901 (3,155)
Femoral neck	-0.50 (0.27)	-0.74 (0.13)	-0.73 (0.39)	-0.74 (0.09)	0.790	0.348 (3,150)
Trochanter	0.04 (0.31)	-0.64 (0.16)	-0.84 (0.30)	-0.59 (0.11)	0.054	2.611 (3,146)
Ward's triangle	-0.21 (0.22)	-0.76 (0.16)	-0.61 (0.45)	-0.70 (0.11)	0.232	1.446 (3,146)

- 15 Only age was significantly different among the groups ($p=0.011$), due to the younger mean age of the males in the second group. However, ANOVA of age as the dependent variable with genotype for the two polymorphisms as class variable indicated that age was unaffected by genotype.
- 20 Therefore the results of genotype testing in the two groups were pooled for subsequent analyses.

The microsatellite repeat within intron 4 of the TNFR2 gene has been reported previously to have 5 alleles (Beltinger et al. *Genomics* 1996 35:94-100). In these test groups, 6 alleles were observed with a statistically significant difference in the frequency of distribution between the two groups ($p<0.025$, Table 2). A total of 18 different genotypes would be expected from 6 alleles assuming Hardy-Weinberg equilibrium. The observed frequency distribution of genotypes of the intron 4 microsatellite repeat for both the test and control groups

-6-

did not differ significantly from expected (Test group Chi-square=20.54, df=19, N.S.; control group Chi-square=10.08, df=19, N.S.). Comparison of the genotype distributions between the test and control groups was also not significant (Chi-square=22.46, df=19, N.S.).

The polymorphism within the 3'UTR of the TNFR2 gene was previously characterized as having 4 alleles that were identified by different banding patterns under electrophoretic conditions that detect single strand conformation differences (Kaufman et al. *Hum. Genet.* 1994 94:418-422; White et al. *Genes, Chrom., Canc.* 1993 7:102-108; and Kaufman et al. *Hum. Molec. Genet.* 1993 2:824). Using a different conformation sensitive gel technique, 5 alleles have now been resolved, each as a single band (Table 2). The allele frequency distribution was compared between the test and control groups, with no statistically significant differences. The frequency distribution of the 15 genotypes was in Hardy-Weinberg equilibrium in both groups (Test group Chi-square= 8.44, df=14, N.S.; Control group Chi-square=22.57, df=14, N.S.). However, comparison of the genotype distributions for the 3'UTR polymorphism between the test and control groups was statistically significant (Chi-square=48.70, df=14, $p<0.001$).

Table 2: Allele frequency distribution for the two TNFR2 polymorphisms in test group (n=159) compared with control group (n=141)

Allele	Intron 4 Polymorphism*		UTR Polymorphism**	
	Test group	Controls	Test group	Controls
1	0.195	0.156	0.059	0.038
2	0.037	0.080	0.229	0.240
3	0.573	0.551	0.154	0.137

-7-

4	0.186	0.193	0.374	0.427
5	0.006	0.004	0.182	0.156
6	0.003	0.015	NA***	NA

*Chi Square = 14.74, df=5, p<0.025

5 **Chi Square = 8.24, df=4, p<0.10

***NA=Not applicable

The nature of the UTR polymorphism was established by sequencing of PCR product from individuals who had been scored as homozygous for alleles 2, 3, 4 and 5 or heterozygous for combination of allele 1 and the other four alleles. No 1/1 homozygotes were observed. The polymorphism is actually a combination of three polymorphic sites within a span of 28 nucleotides, affecting nucleotides 593, 598 and 620 of exon 10 of the TNFR2 gene (Genbank Accession #U52165; SEQ ID NO:1). The five observed alleles are defined as summarized in Table 3.

Table 3: Alleles of the TNFR2 UTR polymorphism as defined by nucleotide composition at three variable sites. Numbering is based on Genbank Accession #U52165

Alleles	Nucleotide 593	Nucleotide 598	Nucleotide 620
1	A	G	T
2	A	T	T
3	G	T	C
4	G	T	T
5	A	T	C

Analysis of variance (ANOVA) was performed on BMD Z-scores at the spine, femoral neck, trochanter and Ward's triangle with respect to genotype at each polymorphic site (Table 4).

Table 4: Results of ANOVA of BMD Z-scores with genotype for each of the two TNFR2 polymorphisms as the grouping variable in the study group (n=159)

	TNFR2 Polymorphism			
	Intron 4 Microsatellite		3'UTR	
	P value	F ratio (df)	P value	F ratio (df)
L2-L4	0.044	1.937 (10,146)	0.170	1.386 (13,145)
5 Femoral Neck	0.120	1.574 (10,141)	0.140	1.459 (13,140)
Trochanter	0.199	1.373 (10,137)	0.127	1.494 (13,136)
Ward's triangle	0.199	1.372 (10,137)	0.149	1.439 (13,136)

10 No statistically significant relationship was observed (i.e. $p < 0.025$). Analysis of the genotype data using Tukey-Kramer post hoc pair-wise comparisons indicated that the mean Z-score for spinal BMD of individuals having genotype 1/2 for the UTR polymorphism was significantly different
 15 from those with genotype 2/2. Analysis of genotype data for the CA polymorphism by the Tukey-Kramer test suggested no significant differences. However, when the UTR genotypes were placed in order with respect to mean spinal BMD Z-scores, it was found that the lowest mean spinal BMD
 20 occurred in the genotype groups that had allele 1, the rarest allele (Table 5).

Table 5: Genotypes for the TNFR2 UTR polymorphism with frequency and mean spinal or femoral neck BMD Z-scores for the study group

25	GENOTYPE	FREQUENCY	SPINAL BMD Z- SCORE* (Mean \pm SE)	FEMORAL NECK BMD Z-SCORE** (Mean \pm SE)
	1/2	5	-2.814 (0.633)	-0.727 (0.469)

5	1/5	4	-1.718 (0.708)	-1.445 (0.469)
	1/3	2	-1.345 (1.00)	-1.620 (0.663)
	1/4	8	-1.316 (0.500)	-0.454 (0.354)
	3/4	21	-1.225 (0.309)	-0.920 (0.215)
	2/3	7	-1.218 (0.535)	-0.494 (0.354)
10	2/4	26	-1.124 (0.277)	-0.777 (0.184)
	2/5	13	-0.980 (0.393)	-0.342 (0.260)
	3/3	6	-0.778 (0.578)	-0.458 (0.419)
	4/4	23	-0.776 (0.295)	-0.868 (0.195)
	3/5	7	-0.750 (0.535)	-1.108 (0.354)
15	5/5	8	-0.719 (0.500)	-1.130 (0.331)
	4/5	18	-0.684 (0.334)	-0.508 (0.221)
	2/2	11	-0.023 (0.427)	-0.022 (0.282)
	Total	159		

*ANOVA, $p=0.17$, $R^2=0.11$

**ANOVA, $p=0.14$, $R^2=0.12$

The mean femoral neck BMD Z-scores are shown for comparison, and indicate a similar trend in that the two lowest mean Z-scores occur in groups having allele 1.

Thus, these analysis show that the frequency of the rarest allele of this gene is associated with low bone density. Further, this association between the genotype of TNFR2 and bone density is statistically significant ($p=0.008$). Accordingly, assessment of the genotype of the TNFR2 gene of an individual is useful in identifying individuals with low bone density at risk for osteoporosis.

In the method of the present invention, a DNA sample is obtained from an individual. The genotype of the TNFR2 gene is then assessed to determine if the individual is at risk for developing osteoporosis. For example, detection of a polymorphisms at nucleotides 593, 598 and 620 of exon 10 of the TNFR2 gene (SEQ ID NO:1) is indicative of an individual at risk of developing osteoporosis.

-10-

The present invention also relates to diagnostic kits comprising a means for identifying individuals at risk of developing osteoporosis based on assessment of the genotype of the TNFR2 gene. In a preferred embodiment, such means
5 are capable of detecting polymorphisms at nucleotides 593, 598 and 620 of exon 10 of the TNFR2 gene (SEQ ID NO:1). Such means can be developed routinely by those skilled in the art based upon known techniques for genotype screening such as PCR and gel electrophoresis. In one embodiment,
10 the kit comprises primers such as exemplified by SEQ ID NO: 4 and SEQ ID NO:5 described in Example 3 herein. However, as will be obvious to those of skill in the art upon this disclosure, additional means useful in assessing the TNFR2 genotype of an individual can also be used. In a preferred
15 embodiment, the kits of the present invention further comprise a blood collection device for obtaining a blood sample from an individual as the source of DNA for genotyping. Other sources of DNA obtained from a patient which can be genotyped via the kits and methods of the
20 present invention include both tissue samples and other biological fluids.

Using the method and/or kits of the present invention, one of skill can screen individuals known to have a family history of low bone density and/or individuals with
25 identified risk factors for osteoporosis and low bone density including, but not limited to, post-menopausal women and the elderly. Via these methods and kits, individuals having polymorphisms in the TNFR2 gene, and in particular polymorphisms detected at nucleotide 593, 598
30 and 620 of exon 10 of the TNFR2 gene (SEQ ID NO:1), can be identified as being at risk of developing osteoporosis.

The following non-limiting examples are provided to further illustrate certain aspects of the instant invention.

EXAMPLES**Example 1: Subjects**

The first group of test subjects was composed of 66 individuals unrelated to each other. These individuals were either the founders of or had married into families with low BMD. The second test group of 93 individuals were also unrelated to each other and to the first group and had been referred to the McGill Bone Center by physicians for evaluation of bone status. The third group was a random general clinic population (n=141), that served as a control for allele frequency distribution in these studies.

Blood was collected from participants and DNA was isolated by standard methods. Bone densitometry was performed on individuals in the first two groups in accordance with procedures described by Devoto et al. *Eur. J. Hum. Genet.* 1998 6:151-157 and Spotila et al. *Calcif. Tissue Int.* 1996 59:235-237.

Example 2: Microsatellite Repeat in Intron 4

The PCR primers for detection of the (CA)₁₆ microsatellite repeat described by Beltinger et al. (*Genomics* 1996 35:94-100) were TNFR2-CAF:5'-GTGATCTGCAAGATGAACTCAC-3' (SEQ ID NO:2) and TNFR2-CAR:5'-ACACCACGTCTGATGTTTCA-3' (SEQ ID NO:3). PCR conditions were denaturation at 94.5°C for 3 minutes; followed by 5 cycles of 94.5°C for 30 seconds, 65.0°C for 30 seconds, 72°C for 30 seconds, followed by 23 cycles of 94.5°C for 30 seconds, 55.0°C for 30 seconds, 72.0°C for 30 seconds; followed by 72.0°C for 5 minutes in a Perkin Elmer Thermocycler 9600. The primer TNFR2-CAF was labeled with γ -³²P-ATP using T4 polynucleotide kinase. Approximately 100-300 ng of DNA template was used with reaction components under conditions recommended by the manufacturer (Applied Biosystems).

Example 3: Polymorphism of 3' Untranslated Region

PCR primers for detection of the polymorphism within

-12-

the 3'UTR of TNFR2 as described by Kaufman et al. (Hum. Genet. 1994 94:418-422) were TNFR2-7:5'-AGGACTCTGAGGCTCTTTCT-3' (SEQ ID NO:4) and TNFR2-8:5'-TCACAGAGAGTCAGGGACTT-3' (SEQ ID NO: 5). PCR conditions were denaturation at 94.5°C for 3 minutes; followed by 28 cycles of 94.5°C for 30 seconds, 62.0°C for 30 seconds, 72.0°C for 30 seconds; followed by 72.0°C for 5 minutes. Reagents and instrumentation were the same as described for Example 2.

Using conformation sensitive gel electrophoresis (CSGE), 5 differentially migrating fragments of this PCR product were distinguished. Gel conditions and product suppliers were similar to those described by Ganguly et al. (Proc. Natl Acad. Sci. USA 1996 93:13721-13725) with the following modifications. Tris-aurine-EDTA running buffer was made as a 20X stock with 1.78 M Tris, 0.58 M Taurine, 4.0 mM EDTA, pH 9.0 and used at 0.5X. Formamide was deionized in batches and frozen in aliquots sufficient for a single gel. Sample loading buffer was prepared each time with 4 parts deionized formamide and 1 part loading buffer (5X=0.1% xylene cyanol, 0.1% bromophenol blue, 10 mM EDTA, 95% formamide). Standard sequencing gel plates and apparatus were used with 0.4 mm spacers. Samples were mixed with an equal volume of sample loading buffer, denatured for 5 minutes at 95°C, transferred to ice and 1.5 µl was immediately loaded. Gels were run at 350 V at room temperature for 16 to 18 hours, then dried and exposed to film. Resolution of 5 alleles was possible only when these conditions were adhered to.

Example 4: Sequence Analysis

Selected PCR fragments were sequenced according to manufacturer's recommendations using ABI 377 instrumentation and Big-Dye chemistry (Applied Biosystems, Inc. Foster City, CA) in order to determine the basis for the 5 alleles.

-13-

Example 5: Statistical Analysis

The two test groups were compared by ANOVA for age, body mass index and BMD Z-score at the spine, femoral neck, trochanter and Ward's triangle, and then combined for additional analyses. The frequency distribution of alleles for the two polymorphisms within the TNFR2 gene was analyzed by Chi Square. Association of bone density at the spine (L2-L4), femoral neck, trochanter and Ward's triangle with genotype for each of the two polymorphisms was evaluated by ANOVA. The BMD Z-scores at each of the anatomical sites were used as the independent variable since they were already corrected for age, gender and weight. Statistical significance was achieved at $p < 0.025$, because of multiple testing. Pairwise comparisons of mean BMD Z-scores for the different genotypes were made for all pairs using the Tukey-Kramer post hoc test. Power analysis was performed to determine the lowest sample number required to observe a significant ($p = 0.025$) effect of genotype on BMD. The statistical software package employed was JMP (Version 3.1, SAS Institute, Inc. Cary, N.C.).

-14-

What is claimed is:

1. A method for identifying individuals at risk of developing osteoporosis comprising assessing the genotype of tumor necrosis factor alpha 2 receptor gene in a sample
5 of DNA from an individual.

2. The method of claim 1 wherein the genotype comprising a polymorphism at nucleotides 593, 598, and 620 in exon 10 of tumor necrosis factor alpha 2 receptor gene (SEQ ID NO:1) is indicative of the individual being at risk
10 for developing osteoporosis.

3. A kit for identifying individuals at risk of developing osteoporosis comprising a means for assessing the genotype of tumor necrosis factor alpha 2 receptor gene in an individual.

15 4. The kit of claim 3 wherein the means detects polymorphisms at nucleotides 593, 598 and 620 in exon 10 of tumor necrosis factor alpha 2 receptor gene (SEQ ID NO:1) which are indicative of the individual being at risk for developing osteoporosis.

Docket No.
DRE-0057

#3

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Methods and Kits for Identifying Individuals at Risk of Developing Osteoporosis

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on 30 November 1999 as United States Application No. or PCT International Application Number PCT/US99/28403 and was amended on _____

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Not Claimed

(Number)

(Country)

(Day/Month/Year Filed)

☐

(Number)

(Country)

(Day/Month/Year Filed)

☐

(Number)

(Country)

(Day/Month/Year Filed)

☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

60/110,268

30 November 1998

(Application Serial No.)

(Filing Date)

(Application Serial No.)

(Filing Date)

(Application Serial No.)

(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*



26259

PATENT TRADEMARK OFFICE

Send Correspondence to:

Direct Telephone Calls to: *(name and telephone number)*

Jane Massey Licata or Kathleen A. Tyrrell - (856) 810-1515

Full name of sole or first inventor

Loretta D. Spotila

Sole or first inventor's signature

Loretta D. Spotila

Date

7/30/01

Residence

Haddonfield, New Jersey *NJ*

Citizenship

US

Post Office Address

161 Merion Avenue

Haddonfield, New Jersey 08033

Full name of second inventor, if any

Second inventor's signature

Date

Residence

Citizenship

Post Office Address

SEQUENCE LISTING

<110> Spotila, Loretta D.
Drexel University

<120> Methods and Kits for Identifying Individuals at Risk of
Developing Osteoporosis

<130> DRE-0019

<140>

<141>

<160> 5

<170> PatentIn Ver. 2.0

<210> 1

<211> 2613

<212> DNA

<213> Homo sapiens

<400> 1

```

tcttggtctc ggctcctggc ccagtgtctt ttcccatgtg tctgaatctg catcttgggc 60
aggggtccct gggccccact cctggacccc cggactgacc cccaccccat cttgtgctta 120
gcagattctt cccctggtgg ccattgggacc caggtcaatg tcacctgcat cgtgaacgtc 180
tgttagcagct ctgaccacag ctcacagtgc tctcccaag ccagctccac aatgggagac 240
acagattcca gcccctcgga gtccccgaag gacgagcagg tccccttctc caaggaggaa 300
tgtgcctttc ggtcacagct ggagacgcca gagaccctgc tggggagcac cgaagagaag 360
cccctgcccc ttggagtggc tgatgctggg atgaagccca gttaaccagg ccggtgtggg 420
ctgtgtcgta gccaaggtgg gctgagccct ggcaggatga ccctgcgaag gggccctggg 480
ccttccaggc cccaccact aggactctga ggctctttct gggccaagtt cctctagtgc 540
cctccacagc cgcagcctcc ctctgacctg caggccaaga gcagaggcag cgagttgggg 600
aaagcctctg ctgccatggg gtgtccctct cggaaggctg gctgggcatg gacgttcggg 660
gcatgctggg gcaagtccct gactctctgt gacctgcccc gccagctgc acctgccagc 720
ctggcttctg gagcccttgg gttttttgtt tgtttgtttg tttgtttgtt tgtttctccc 780
cctgggctct gccagctct ggcttccaga aaaccccagc atccttttct gcagaggggc 840
tttctggaga ggagggatgc tgcttgagtc acccatgaag acaggacagt gcttcagcct 900
gaggctgaga ctgcgggatg gtcttggggc tctgtgtagg gaggagggtg cagccctgta 960
gggaacgggg tccttcaagt tagctcagga ggcttggaag gcatcacctc aggccagggtg 1020
cagtggctca cgcctatgat ccagcactt tgggaggctg aggcgggtgg atcacctgag 1080
gttaggagtt cgagaccagc ctggccaaca tggtaaaacc ccattctctac taaaaataca 1140
gaaattagcc gggcgtgggt gcgggcacct atagtccag ctactcagaa gcctgaggct 1200
gggaaatcgt ttgaaccggg gaagcggagg ttgcaggag cagagatcac gccactgcac 1260
tccagcctgg gcgacagagc gagagtctgt ctcaaaagaa aaaaaaaaaa gcaccgcctc 1320
caaatgctaa cttgtccttt tgtaccatgg tgtgaaagtc agatgccag agggccagg 1380
caggccacca tattcagtgc tgtggcctgg gcaagataac gcacttctaa ctagaaatct 1440
gccaatTTTT taaaaaagta agtaccactc aggccaacaa gccaacgaca aagccaaact 1500

```

```

ctgccagcca catccaaccc cccacctgcc atttgacccc tccgccttca ctccggtgtg 1560
cctgcagccc cgcgcctcct tccttgctgt cctaggccac accatctcct ttcagggaat 1620
ttcaggaact agagatgact gagtccctgt agccatctct ctactcctac ctcagcctag 1680
accctcctcc tccccagag gggtaggttc ctcttcccca cccccacct tcaattcctg 1740
ggccccaac gggctgcct gccacttttg tacatggcca gtgtgatccc aagtgccagt 1800
cttgtgtctg cgtctgtgtt gcgtgtcgtg ggtgtgtgta gccaaagtcg gtaagttgaa 1860
tggcctgcct tgaagccact gaagctggga ttctcccca ttagagtcag ccttccccct 1920
cccagggcca gggccctgca gaggggaaac cagtgtagcc ttgcccggat tctgggagga 1980
agcaggttga ggggctcctg gaaaggctca gtctcaggag catggggata aaggagaagg 2040
catgaaattg tctagcagag caggggcagg gtgataaatt gttgataaat tccactggac 2100
ttgagcttgg cagctgaact attggagggt gggagagccc agccattacc atggagacaa 2160
gaagggtttt ccacctgga atcaagatgt cagactggct ggctgcagt acgtgcacct 2220
gtactcagga ggctgagggg aggatcactg gagcccagga gtttgaggct gcagcgagct 2280
atgatcgcgc cactacactc cagcctgagc aacagagtga gaccctgtct cttaaagaaa 2340
aaaaaagtca gactgctggg actggccagg tttctgcca cattggaccc acatgaggac 2400
atgatggagc gcacctgccc cctggtggac agtcctggga gaacctcagg cttccttggc 2460
atcacagggc agagccggga agcgatgaat ttggagactc tgtggggcct tggttccctt 2520
gtgtgtgtgt gttgatccca agacaatgaa agtttgcact gtatgctgga cggcattcct 2580
gcttatcaat aaacctgttt gttttaaaaa aaa 2613

```

<210> 2

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic

<400> 2

gtgatctgca agatgaactc ac

22

<210> 3

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic

<400> 3

acaccacgtc tgatgtttca

20

<210> 4

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic

<400> 4

aggactctga ggctctttct

20

<210> 5

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic

<400> 5

tcacagagag tcagggactt

20